Role of the \(\alpha_2\)-isoform of AMP-activated protein kinase in the metabolic response of the heart to no-flow ischemia

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Adenosine 5’-monophosphate-activated protein kinase (AMPK), a ubiquitous serine/threonine protein kinase, senses the energy state of the cell. AMPK is a heterotrimeric enzyme consisting of a catalytic (\(\alpha\)) and two regulatory (\(\beta\) and \(\gamma\)) subunits. Different isoforms for each of these subunits have been identified (43). In the heart, the catalytic \(\alpha_2\)-subunit is predominant and represents 60–70% of total AMPK activity; the remaining activity is due to the \(\alpha_1\)-subunit (9). Control of AMPK activity is complex and involves allosteric stimulation by AMP as well as AMP-dependent phosphorylation at Thr172, a residue located in the activation loop of the \(\alpha_2\)-subunit (15). Several protein kinases responsible for this phosphorylation have been identified: LKB1 (33, 39, 50) and the Ca\(^{2+}\)/calmodulin-dependent protein kinase kinase (16, 24, 49). The rapid rise in AMP concentration during an ischemic episode explains the activation of cardiac AMPK under this pathological condition (21, 22). Moreover, AMPK can also be activated by antidiabetic drugs (12, 55), osmotic stress (11), leptin (35), and adiponectin (53), probably via AMP-independent pathways. In hearts subjected to ischemia-reperfusion, AMPK can be considered a metabolic master switch (21, 42, 54). Indeed, once activated, AMPK phosphorylates several downstream targets, switching on ATP-generating pathways and switching off ATP-consuming biosynthetic pathways, thereby moderating the negative effects of ischemia-reperfusion on the heart’s energy balance (14). During ischemia, AMPK promotes glycolysis by a dual mechanism: 1) it increases glucose uptake by stimulating translocation of GLUT4 transporters to the sarcolemmal membrane (37) and 2) it indirectly stimulates 6-phosphofructo-1-kinase (PFK-1) activity by phosphorylating and activating the heart isoform of 6-phosphofructo-2-kinase, the enzyme that synthesizes fructose 2,6-bisphosphate, a potent PFK-1 stimulator (34). During early reperfusion, AMPK is involved in the dominance of fatty acid oxidation over glucose oxidation. AMPK phosphorylates and inactivates acetyl-CoA carboxylase (ACC), thereby decreasing the concentration of malonyl-CoA, the inhibitor of fatty acid transport into mitochondria, and increasing fatty acid oxidation (31). Recent studies also implicate AMPK in the inhibition of protein synthesis during anaerobic conditions. Indeed, AMPK inactivates eukaryotic elongation factor 2 (eEF2) by phosphorylating and activating eEF2 kinase, the upstream kinase responsible for phosphorylation and inactivation of eEF2 (6, 17, 18). Moreover, AMPK plays a role in regulation of the mammalian target of rapamycin (mTOR), a protein kinase controlled by hormonal and nutritional status and involved in cell growth (2). It has been recently shown that AMPK can inactivate mTOR by direct phosphorylation of the enzyme (5) and phosphorylation of the tuberous sclerosis complex 2, an upstream regulator of mTOR (25). In previous studies, the metabolic role of AMPK has most often been evaluated by use of mitochondrial poisons, such as oligomycin, or pharmacological AMPK activators, such as 5-aminimidazole 4-carboxamide \(\beta,\beta\)-ribo-
furanoside or antidiabetic drugs. The results of these studies should be interpreted with caution, because these agents are not totally specific for AMPK. Recently, transgenic mice overexpressing a dominant-negative form (K45R mutation) of AMPKα2 (DN-K45R) in the heart have been studied during low-flow ischemia (38). These mice are characterized by a loss of AMPKα1 and AMPKα2 stimulation, glucose uptake, and fatty acid oxidation during ischemia-reperfusion. Accordingly, hearts of these mice exhibit impaired recovery of left ventricular (LV) function during reperfusion. In the present study, we used mice in which only the catalytic AMPKα2 gene was inactivated (AMPKα2−/− mice) (48). These mice allowed us 1) to study the specific role of AMPKα2 in the control of heart metabolism during no-flow ischemia and 2) to compare the data obtained with these AMPKα2−/− mice with those reported in other transgenic models.

MATERIALS AND METHODS

This study was approved by the Animal Research Committee at Université catholique de Louvain and conformed to the American Heart Association Guidelines for Use of Animals in Research.

Echocardiographic analysis. Two-dimensional echocardiography was performed with a Sonos 7500 system equipped with a 15-MHz linear-array transducer (Phillips Medical System, Eindhoven, The Netherlands). LV function was assessed in tribromoethanol-anesthetized (Avertin, Fluka; 0.3 mg/g body wt ip) mice from short-axis (22) and long-axis (23) views. The echocardiographic examination was performed by one investigator (F.G.) who did not know the genotypes of the animals. The size of the LV was measured from the M-mode tracings, and the following parameters were calculated: end-systolic (ES) and end-diastolic (ED) dimensions, fractional shortening (FS), and ejection fraction (EF). The EF was calculated as EF = (ED − ES)/ED × 100. Results are expressed as means ± SE. WT, wild type; AMPKα2−/− and wild-type (WT) mice were similar (data not shown).

Results of the echocardiographic examinations. In contrast to the results obtained in dominant-negative AMPK transgenic mice (38), LV functional parameters were not significantly different between hearts from AMPKα2−/− and WT mice. In particular, no signs of LV hypertrophy, LV dilation, or heart failure were noted.

Expression of α- and β-subunits of AMPK in AMPKα2−/− mice. As expected, immunoblot analysis of myocardial protein extracts of hearts from AMPKα2−/− mice revealed no significant amount of the native α2-isooform of AMPK, whereas the level of AMPKα1 was comparable to that in WT mice (Fig. 1). In contrast to skeletal muscle and liver, a band corresponding to a truncated and inactive AMPKα2 (resulting from deletion of amino acids 189–260 matching the AMPK catalytic domain) could clearly be identified on the immunoblots (Fig. 1).

Metabolites and protein measurements. AMP, ADP, and ATP levels were measured in neutralized perchloric acid extracts of the frozen hearts after their separation by high-performance liquid chromatography (47). Lactate was measured enzymatically in extracts described previously (3). For evaluation of glycogen content, frozen hearts were homogenized in 10 vol (vol/wt) of 1 M KOH. The homogenates were heated (80°C, 15 min), chilled on ice, neutralized (0.25 vol of 3.3 M acetic acid, pH 6, 10 min), and then centrifuged (5,000 g, 4 min). Glycogen content was measured in the supernatants as previously described (23). Protein was estimated by the method of Bradford, with bovine serum albumin as a standard.

Table 1. Echocardiographic analysis

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>AMPKα2−/−</th>
<th>P</th>
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<tr>
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<tr>
<td>AW thickness, mm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diastole</td>
<td>0.72±0.04</td>
<td>0.68±0.03</td>
<td>NS</td>
</tr>
<tr>
<td>Systole</td>
<td>1.46±0.06</td>
<td>1.39±0.07</td>
<td>NS</td>
</tr>
<tr>
<td>AW thickness, %</td>
<td>50.4±2.2</td>
<td>50.7±3.2</td>
<td>NS</td>
</tr>
<tr>
<td>LVED, mm</td>
<td>3.36±0.21</td>
<td>3.45±0.25</td>
<td>NS</td>
</tr>
<tr>
<td>ES</td>
<td>1.59±0.11</td>
<td>1.58±0.25</td>
<td>NS</td>
</tr>
<tr>
<td>FAC, %</td>
<td>52.6±4.0</td>
<td>54.3±2.5</td>
<td>NS</td>
</tr>
<tr>
<td>LV area, mm²</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ED</td>
<td>8.77±0.86</td>
<td>8.33±1.00</td>
<td>NS</td>
</tr>
<tr>
<td>ES</td>
<td>2.66±0.35</td>
<td>2.54±0.48</td>
<td>NS</td>
</tr>
<tr>
<td>EF, %</td>
<td>69.6±3.6</td>
<td>69.2±6.0</td>
<td>NS</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>345±32</td>
<td>330±24</td>
<td>NS</td>
</tr>
</tbody>
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Values are means ± SE. WT, wild type; AMPKα2−/−, AMP-activated protein kinase-α2 knockout; AW, anterior wall; LVD, left ventricular (LV) distance; ED, end diastole; ES, end systole; EF, ejection fraction; FAC, fractional area changes; HR, heart rate. There is no significant difference between WT and AMPKα2−/− mice for any parameter.
AMPKβ, which binds to AMPKα and AMPKγ and, therefore, is essential for formation of the heterotrimer (7, 14), has been recently shown to be involved in binding of AMPK to glycogen (20, 36, 40). Immunoblots revealed a dramatic decrease in AMPKβ2, the major β-subunit in the heart (44), in AMPKα2−/− mice (Fig. 1). Immunodetection with an anti-pan AMPKβ antibody showed the same profile, eliminating possible compensation by an increase in AMPKα1 (Fig. 1).

**AMPK activity during normoxia and ischemia.** The effect of ischemia on AMPK activity was evaluated in Langendorff-perfused hearts from AMPKα2−/− mice subjected to 10 min of normoxia or no-flow ischemia. In WT mice, no-flow ischemia induced a fivefold increase in AMPKα2 activity (Fig. 2A). As expected, in hearts from AMPKα2−/− mice, no AMPKα2 activity could be detected during normoxia and ischemia. Because the same antibody was used for activity measurement and immunoblotting, these results indicate that the truncated form of AMPKα2, visible in Fig. 1, is indeed inactive. In contrast, AMPKα1 activity was not different during normoxia and increased four- to fivefold after 10 min of ischemia in WT and AMPKα2−/− mice (Fig. 2B). Finally, the absence of the active AMPKα2 resulted in a significant decrease (50%) in the activation of total AMPK by no-flow ischemia, as measured by polyethylene glycol fractionation (Fig. 2C). Similarly, it induced a significant decrease in total AMPK phosphorylation at Thr172 (data not shown).

**Nucleotide content.** Cardiac AMPK activation during ischemia has been shown to result from an increase in the AMP-to-ATP ratio (21, 22). A clear correlation between the level of AMPK activation and the increase in the AMP-to-ATP ratio after 10 min of ischemia has indeed been reported in WT animals (3, 34). To evaluate whether this relation is preserved in hearts from AMPKα2−/− mice, the nucleotide content of perfused hearts was measured during normoxia and ischemia. In WT mice, no-flow ischemia induced a threefold increase in AMP content, whereas ATP concentration was slightly, but not significantly, diminished (Fig. 3), thereby causing a threefold increase in the AMP-to-ATP ratio (Fig. 3; P < 0.05, unpaired t-test) as previously described in rat heart (3). In hearts from AMPKα2−/− mice subjected to ischemia, these changes were even more pronounced (Fig. 3), inasmuch as the AMP content increased 10-fold and the ATP content decreased 5-fold, resulting in an 80-fold increase in the AMP-to-ATP ratio.

**Fig. 1.** Western blot of AMP-activated protein kinase (AMPK)α1, AMPKα2, AMPKβ2, and pan AMPKβ proteins in perfused hearts from wild-type (WT, +/+), AMPKα2-knockout (AMPKα2−/−, −/−), and AMPKα2+/− (+/−) mice during normoxia (N) and ischemia (I). Total eukaryotic elongation factor 2 (eEF2) was used as loading control (data not shown).

**Fig. 2.** Cardiac AMPKα2 (A), AMPKα1 (B), and total AMPK (C) activities during normoxia (open bars) and ischemia (solid bars) in hearts from WT and AMPKα2−/− mice. Values are means ± SE of ≥7 hearts. *P < 0.05; **P < 0.01 (unpaired t-test).
Lactate and glycogen content. To evaluate possible mechanisms underlying the decrease in ATP content in hearts of AMPKα2−/− mice subjected to ischemia, glycogen and lactate cardiac tissue levels were measured during normoxia and ischemia. In WT mice, ischemia resulted in a 4-fold decrease in glycogen content and a 15-fold increase in lactate concentration (Fig. 4). In contrast, glycogen content was fourfold lower in hearts from AMPKα2−/− mice than from WT mice during normoxia and was barely detectable after ischemia. Accordingly, lactate accumulation was three times less in AMPKα2−/− than in WT mice.

LV function. The evolution of LV functional parameters during equilibration, ischemia, and reperfusion is shown in Figs. 5 and 6. During normoxia, the rate-pressure product, representing the index of contractility, was similar among WT and AMPKα2−/− mice (Fig. 6). During no-flow ischemia, ischemic contracture developed in both groups (Fig. 5). However, the onset of contracture and the time to its maximal amplitude were reduced three- and twofold, respectively, in AMPKα2−/− mice compared with WT mice. Peak diastolic pressure was also significantly higher in hearts from AMPKα2−/− than WT mice.

Although the onset of ischemia-induced contracture is more rapid in hearts from AMPKα2−/− mice, both groups are characterized by the same functional recovery on reperfusion (Fig. 6). Indeed, the resulting decrease in rate-pressure product and oxygen consumption was similar in WT and AMPKα2−/− mice, whereas coronary flow was not statistically modified in either group of animals.

Downstream targets of AMPK. To determine whether the absence of myocardial AMPKα2 impairs phosphorylation of downstream substrates of AMPK during ischemia, we measured ACC phosphorylation in hearts perfused during normoxia and ischemia. ACC, one of the first AMPK substrates identified, was clearly phosphorylated (80% increase) during ischemia in WT mice (Fig. 7). In hearts from AMPKα2−/− mice, ischemia also induced ACC phosphorylation, although it was three to five times less than in WT mice. Accordingly, the ACC phosphorylation state in ischemic hearts from AMPKα2−/− mice was two times less than in normoxic WT mice (Fig. 7), despite the presence of AMPKα1 in the AMPKα2−/− mice.

DISCUSSION

Cardiac phenotype of AMPKα2−/− mice. We have shown that the absence of AMPKα2 does not result in any detectable

Fig. 3. AMP and ATP contents and AMP-to-ATP ratio in perfused hearts from WT and AMPKα2−/− mice during normoxia (open bars) and ischemia (solid bars). Total amount of adenine nucleotides of each animal was measured (not shown), and no significant differences were observed between WT and AMPKα2−/− mice. Values are means ± SE of ≥8 hearts. *P < 0.05; **P < 0.01 (unpaired t-test).

Fig. 4. Glycogen and lactate contents of perfused hearts from WT and AMPKα2−/− mice during normoxia (open bars) and ischemia (solid bars). Values are means ± SE of ≥8 hearts. **P < 0.01 (unpaired t-test).
morphological or functional changes in the heart. Indeed, heart size and echocardiographic and hemodynamic parameters under basal conditions were similar among hearts from WT and AMPKα2−/− mice. In contrast, in DN-K45R transgenic mice, a smaller heart and a statistically significant decrease in cardiac contractility (decreased maximum rate of pressure increase and fractional wall thickening) were recently reported (38). Although this remains speculative, the differences between DN-

Fig. 5. Sensitivity of heart function to no-flow ischemia. Perfused hearts of WT (open bars) and AMPKα2−/− (solid bars) mice were subjected to 15 min of normoxia (15 min) followed by no-flow ischemia. Heart function was followed during the entire period of perfusion (up to 31 min). A: left ventricular pressure recording from a representative experiment. B: time corresponding to beginning of ischemic contracture and time and amplitude of maximal contracture (maximal end-diastolic pressure [EDP]). Values are means ± SE of 3 hearts. **P < 0.01 (unpaired t-test).

Fig. 6. Time-dependent response of function parameters during ischemia-reperfusion protocol. After equilibration in normoxic conditions, perfused heart of WT (+) and AMPKα2−/− (●) mice were subjected to 30 min of no-flow ischemia (from −30 to 0 min) followed by 45 min of reperfusion (from 0 to 45 min). Heart rate, left ventricular systolic pressure, end-diastolic pressure, and coronary flow were measured online. Contractility was estimated as rate-pressure product. Oxygen consumption was measured online from the difference in oxygen content between incoming (aortic) and outgoing (pulmonary artery) perfusate and expressed in μmol O2·min−1·g wet wt−1.
that the correlation between the AMP-to-ATP ratio and AMPK activation established in WT hearts is modified in hearts from AMPKα2−/− mice. One explanation for this difference could be that AMPKα1 is less sensitive than AMPKα2 to AMP (41).

**Glycolysis, glycogen, and AMPKβ.** Our data indicate that the ATP depletion characteristic of ischemic hearts from AMPKα2−/− mice most probably results from a reduction in ATP production by glycolysis as measured by lactate production. Indeed, ATP production is known to be correlated to glucose utilization by anaerobic glycolysis, the sole energy-providing pathway in the absence of oxygen (21, 22). Although several mechanisms could account for the decrease in ATP production, including the loss of the stimulating effect of AMPK on glycolysis [via activation of PFK-2 (34)], our data suggest that preischemic glycogen depletion (5 and 20 mg/g of protein in hearts from AMPKα2−/− and WT mice, respectively) was the most likely cause of the decrease in lactate production.

There are several potential explanations for the lower glycogen content in hearts from AMPKα2−/− mice during normoxia. 1) Defective cardiomyocytes from AMPKα2−/− mice, which are less able to take up exogenous glucose, may have less glycogen-storing capacity than WT cardiomyocytes. Previous studies have indeed shown that AMPK activation is important for translocation of the glucose transporter GLUT4 to the plasma membrane (37). Moreover, this mechanism is indeed deficient in hearts from AMPKα2−/− mice and contributes to their reduced ability to increase exogenous glucose uptake as, for instance, during normoxia and low-flow ischemia (unpublished observations). 2) Several recent studies have shown that AMPK is physically linked to glycogen, probably via AMPKβ (20, 36, 40). Although the exact role of this glycogen-AMPK interaction remains to be elucidated, it is somehow tempting to hypothesize that the decrease in AMPKβ2 in hearts from AMPKα2−/− mice interfered with the ability of AMPK to bind glycogen and, thereby, contributed to disruption of its glycogen-storing capabilities. Alteration of glycogen content, a hallmark of several AMPK isofrom mutations, reinforces this hypothesis (7, 36, 54) and further suggests that the mere decrease in the level of AMPKβ2 in hearts from AMPKα2−/− mice could explain the perturbation of glycogen storage. 3) We cannot exclude the possibility that a modification of glycogen synthase or glycogen phosphorylase activity contributed as well. Indeed, recently, it was reported that AMPKα2 is a glycogen synthase kinase in skeletal muscle (26).

Very recently, we studied the effect of cardiac deletion of LKB1, one of the putative upstream AMPK kinases, on ischemia-induced AMPK activation and nucleotide content (39). Under the same experimental protocol, the absence of LKB1 in the heart induced total abolition of AMPKα2 activity during normoxia and ischemia, whereas AMPKα1 activity was partially decreased (50% compared with WT animals). So, in terms of AMPK activity, a more dramatic phenotype was induced in hearts from LKB1−/− than from AMPKα2−/− mice. In contrast to hearts from AMPKα2−/− mice, the increase in the AMP-to-ATP ratio during ischemia is, however, only slightly affected by the lack of LKB1 (39). This implies that the presence of AMPKα2 protein, which is not affected by the absence of LKB1, is more important than its activity in preservation of ATP and, probably, glycogen content.
Our study was performed with glucose as the sole energy-providing substrate. We intentionally perfused hearts without fatty acids or insulin, two molecules usually found in vivo, to avoid any possible interference due to the nature (type of fatty acids) and concentration of these molecules. It has been shown that fatty acids and insulin can modify AMPK activity (3, 4, 10, 19). Furthermore, it is difficult to mimic the in vivo situation, inasmuch as fatty acid concentration considerably fluctuates in vivo (42). One could, however, argue that our glucose-only perfusion protocol could have resulted in partial energy starvation and, thereby, contributed to artificially reduced preischemic glycogen levels. However, this is unlikely, because normal normoxic levels of glycogen and nonmodified ischemic ATP levels were found in WT hearts (Figs. 3 and 4). It would nonetheless be interesting to compare our results with those of similar studies performed with different substrates or during low-flow ischemia.

Early LV contracture but normal postischemic contractile function recovery. In isolated perfused hearts, prolonged ischemia almost invariably results in the appearance of ischemic contracture. This rise in ED pressure is due to the lack of ATP at the myofibrillar level, which maintains the cross bridges in the attached state (46). In the present study, ischemic contracture appeared four times more rapidly in hearts from AMPKα2−/− mice from WT mice, probably as a result of a faster and more important decrease in ATP content, in contrast to a more ancient transgenic dominant-negative AMPKα2 (D157A mutation) mouse (52). In these mouse hearts, which were perfused following a protocol similar to that used in this study (heart perfused without fatty acids or insulin and subjected to 10 min of no-flow ischemia), ischemic contracture and ATP depletion appeared only slightly more rapidly than in WT hearts. There are several possible explanations for the different behavior of D157A transgenic mice and AMPKα2−/− mice. 1) AMPKα2 activity was completely blunted in our model, whereas significant activity persisted in the D157A transgenic mice. 2) Glycogen content was markedly reduced in AMPKα2−/− mice, whereas it was unaffected in D157A transgenic mice. 3) As mentioned above, AMPKβ2 content was reduced in our model.

Nevertheless, hearts from AMPKα2−/− mice were characterized by a faster appearance of ischemic contracture and less stimulation of anaerobic glycolysis. We (45) and others (1, 28) previously demonstrated that the rate of glycolysis during ischemia was the most important determinant of the ischemic contracture. The present study of hearts from AMPKα2−/− mice confirms the previously established relation between glycolytic rate and ischemic contracture. In contrast, the usual relation between intensity of ischemic contracture and impairment of postischemic contractility is clearly not present in the AMPKα2−/− mouse model. Indeed, AMPKα2 deletion did not aggravate the contractile consequences induced by no-flow ischemia, notwithstanding acceleration of the ischemic contracture. Similar observations were also made under low-flow ischemia (unpublished observations). The same dichotomy between acceleration of ischemic contracture and recovery of contractile function has been found in WT animals after preconditioning (29, 30). Our results seem to reveal a dual effect of AMPKα2: a positive effect (e.g., glycogen storage and stimulation of glucose) during normoxia and ischemia and a negative effect during reperfusion. Several hypotheses could explain this putative deleterious effect of AMPK during reperfusion. 1) AMPK, via ACC inactivation, is known to increase posts ischemic fatty acid oxidation. This fatty acid oxidation induces uncoupling of glycolysis and glucose oxidation and, therefore, participates in myocardial reperfusion injury (42). Even if our perfusion protocol (with glucose as the sole substrate) excludes a role for exogenous fatty acid, the remaining endogenous fatty acid could participate in this deleterious effect, which should be less pronounced in hearts from AMPKα2−/− mice, characterized by a lower ACC phosphorylation (see below). 2) The energetic cost of contractility has been found to be modified in hearts from AMPKα2−/− mice (unpublished observations). This could also be a factor in the postischemic recovery of contractile function that characterizes these hearts.

Our results are different from those reported by Russell et al. (38) from DN-K45R transgenic mouse hearts, because 1) they used fatty acid as the alternative substrate, 2) the remaining AMPKα1 in our model could compensate for the absence of AMPKα2, and 3) overexpression of a dominant-negative form of AMPK can induce side effects (see above).

Metabolic consequences under normoxia and ischemia. Hearts from AMPKα2−/− mice are also characterized by reduced phosphorylation of ACC during normoxia and no-flow ischemia. Even if AMPKα1 is still present and is probably responsible for the remaining stimulation of ACC phosphorylation during ischemia, the level of phosphorylated ACC after 10 min of ischemia in hearts from AMPKα2−/− mice was smaller than the level during normoxia in control hearts. It is likely that the general decrease in ACC phosphorylation in hearts from AMPKα2−/− mice would reduce the stimulation of fatty acid oxidation during reperfusion, as in DN-K45R transgenic mice (38).

In conclusion, our results demonstrate that the absence of AMPKα2 is responsible for several alterations in myocardial metabolism during normoxia and ischemia: a reduced ability to store glycogen and stimulate glycolysis, an impaired regulation of ATP homeostasis, and a decrease in the stimulation of fatty acid oxidation. The presence of AMPKα1 does not compensate for the absence of AMPKα2. Despite an apparent worse metabolic adaptation during ischemia, the absence of AMPKα2 does not exacerbate the impairment of posts ischemic recovery of contractile function. This is the first study that reveals a specific role for one of the two catalytic α-subunits of AMPK in the heart.
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